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Development of a rapid and reliable bioassay to discriminate between susceptible and resistant cultivars of tomato against Fusarium wilt

Pritesh. P. Parmar[1], Vishal. P. Oza[1], A. D. Patel[2], K. B. Kathiria[2] and R.B. Subramanian[1]
1 B.R.D.School of Biosciences, Sardar Patel University, Post Box no. 39, V. V. Nagar, 388120 Gujarat INDIA
asp.fus@gmail.com
2 Main Vegetable Research station, Anand Agriculture University, Anand, 388001 (Gujarat) INDIA

ABSTRACT:
A rapid and reliable detached leaf bioassay was developed to identify resistant and susceptible cultivars of tomato against *Fusarium oxysporum* f. sp. *lycopersici* (FOL), in ten different tomato cultivars by dip inoculation method. Among the ten cultivars used for the study, a wide variation for wilt resistance was observed. Six tomato cultivars were highly susceptible, and four were highly resistant. The bioassay significantly discriminated between susceptible and resistant cultivars. *In vivo* assay carried out in field as well as quantification of stress related enzymes and chlorophyll content validated the reliability and sensitivity of the bioassay.

Keywords: Bioassay; Fusarium, Resistance, Tomato

INTRODUCTION

Tomato is considered as one of the world's most important and popular vegetables. Many constraints affect the productivity and quality of tomato among which disease play a salient role. Tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* which is a fungus that become one of a limiting factor in the production of tomato and accounts for yield losses annually.

Fusarium oxysporum f.sp. *lycopersici* (Sacc.) Snyder and Hansen (*Fol*), was first described by Massee (1895) as the 'sleepy disease'. This fungus belongs to the *Hyphomycetes* and was first reported in Europe (Italy) by Petri in 1933 [1]. As a soil-borne fungus, *Fol* enters tomato plants through the roots, and can infect tomato plants at all growth stages. The fungus grows in the vascular bundles and inhibits water flow causing wilting, ultimately leading to plant death [2]. In severe cases it may cause 80% loss in tomato production [3].

The identification and utilization of tomato plant varieties resistant to the disease represents a valid alternative to the use of chemicals. Evaluation of resistance in a short span of time with minimal cost under uniform infestation by the pathogen is necessary for screening large populations of plants for resistance [4]. *In vitro* bioassays are very useful tools to determine the level of resistance in a breeding programme or for screening a population for sensitivity to a pathogen or pathogen derived metabolites [5]. Bioassays play a key role in the rapid screening for evaluating resistance and susceptibility of a plant species against the pathogen [6]. Rapid and reliable bioassays are essential for evaluation of both natural and induced resistance. Present investigation is a step towards establishment of a method for quick discrimination of susceptible and resistant cultivars with ease with minimum requirements.

MATERIALS AND METHODS

The ten cultivars evaluated in this study were Anand 1, Heamsona, Junagadh Ruby, Gujarat Local 1, Gujarat Local 2, LA 3042, Maha-2, NDT 96, Pusa Ruby and SS-NC. Cultivar LA 3042 (Tomato genetics resource centre, UC) was used as a resistant check and Pusa ruby (Anand Agriculture University, Anand) a susceptible check.

Inoculum preparation

Fusarium oxysporum f.sp. *lycopersici* race 1 culture obtained from Indian Type Culture Collection, I.A.R.I, New Delhi (F-1322), was maintained on potato dextrose agar at 26±2^{0}C for 96 hrs. Fungal culture filtrate was prepared by inoculating Potato dextrose broth and incubated at 26^{o}C under static condition in dark for 10 days. The liquid was filtered through four layers of cheesecloth and Whatman filter no. 1 to remove mycelia. The culture filtrate obtained was centrifuged at 3000 g for 30 minutes to sediment spores. The supernatant was used and spore pellet was decanted.

Plant material

From all ten cultivars, leaves positioned laterally at the second node were collected from the apex of sixty days old plants grown in open field, washed under running water and surface sterilized by dipping into 1% sodium hypochlorite solution for 3 minutes, followed by sterile distilled water rinse. Leaves of all ten cultivars were then punched to a 1 cm disc and transferred to Petri dishes containing 1% basal agar medium with the help of a sterile forceps under aseptic conditions. For each cultivar, two sets of Petri plates were set. The experimental set of Petri dishes were flooded with the 15 X Fungal culture filtrate (FCF) solution in experimental plates while the control plates were flooded with potato dextrose broth and incubated at room temperature. The leaf discs were assessed after

24, 48, 96 hrs and green leaf area deterioration evaluated qualitatively and quantitatively.

Chlorophyll Evaluation

Quantification of deteriorated area or chlorosis was performed by Witham [7]. After 48 hrs, leaf discs were blotted dry with the help of tissue paper. The representative discs were then weighed to 1g, cut and homogenized to a fine pulp with 20ml of 80% acetone in a chilled mortar and pestle. The pulp was then centrifuged at 5000 g for 5 min and supernatant was transferred to a 100 ml volumetric flask. This process was repeated until the residue became colorless. Mortar and pestle was washed thoroughly with 80% acetone and the clear washing was collected in a 100 ml flask. The absorbance of reaction mixture was taken at 645 nm, 663 nm, and 652 nm, after finalization of volume to 100 ml in volumetric flask by 80% acetone.

Assay of Peroxidase (EC. 1.11.1.7)

Using a chilled pestle and mortar, 1 g of leaf sample was homogenized in 3ml of 0.1 M potassium phosphate buffer (pH 7.0), containing 0.05 mM phenyl methane sulfonyl fluoride (PMSF) to which a pinch of polyvinylpyrollidone (PVP) was added. The homogenate was centrifuged at 8000 g for 15 min at 4^oC and the supernatant was used as the enzyme source for the assay of PO activity.

The reaction mixture consisting of 1.0 ml of 0.01 M O-Dianisidine, 2.4 ml of distilled water, 1.0 ml of 0.1 M phosphate buffer and 0.5 ml of 20mM H_2O_2. The reaction was initiated by 0.2 ml of enzyme extract and the mixture was incubated at room temperature. After 5 minutes reaction was terminated by application of 1.0 ml 2 N H_2SO_4 and absorbance were taken at 430 nm in a spectrophotometer. At the start of enzyme reaction, the absorbances of the control mixture containing 1.0 ml of 0.01 M O-Dianisidine, 2.4 ml of distilled water, 1.0 ml of 0.1 M phosphate buffer and 0.5 ml of 20 mM H_2O_2, 0.2 ml of enzyme extract and 1.0 ml 2 N H_2SO_4 was set to zero at 430 nm in a spectrophotometer. PO activity was expressed as change in the absorbance of the reaction mixture min^{-1} $g^{-1}ml^{-1}$ of fresh weight [8].

Assay of Poly Phenol Oxidase (EC. 1.14.18.1)

PPO activity was assayed using the modified method of Mayer [9]. The standard reaction mixture contained 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0), 0.5 ml of enzyme preparation and 0.5 ml of 0.1 M catechol. The reaction mixture was incubated at room temperature and the absorbance was set to zero at 495 nm. The change in the absorbance was recorded at 30 sec intervals for two min and the PPO activity was expressed as change in absorbance of the reaction mixture ml^{-1} g^{-1} of fresh weight.

Phenolic content

Phenolic content of tomato leaves was estimated as per the procedure given by Zielson and Ben- Zaken [10]. One gram of leaf tissue was homogenized in 10 ml of methanol (80 %) and agitated for 15 min at 70 ^oC. One ml of this homogenate was added to 5 ml of distilled water and 250µl of Folin ciocalteau reagent (1N) was added and the solution was kept at 25^oC. After three minutes, 1 ml of saturated solution of Na_2CO_3 and 1 ml of distilled water was added and the reaction mixture was incubated for 1 hr at 25^oC. The blue colour developed was measured using a spectrophotometer (Unicam ∞) at 725 nm. The quantity of total soluble phenols was calculated using a standard curve obtained from a Folin ciocalteau reaction with Catechol solution and expressed as phenol equivalent in µgm g^{-1} fresh tissue

β- 1,3 glucanase (EC. 3.2.1.39)

β-1, 3 Glucanase activity was assayed by the laminarin Dinitrosalicyclate method [11]. One gram of Tomato leaf was homogenized with 3ml of sodium acetate buffer (0.05 M), pH 5.0 at 4^0C using a chilled pestle and mortar. The extract was then centrifuged at 10,000 g for 15 min at 4^0C and the supernatant was used as crude enzyme extract. The crude enzyme extract (62.5 µl) was added to an equal volume of laminarin (4%) and incubated at 40^0C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent and boiled for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of water, vortexed and absorbance at 500 nm was determined. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µmol min^{-1} ml^{-1}.

PAL Activity (EC. 4.3.1.5)

PAL activity was determined as the rate of formation of phenylalanine to trans-cinnamic acid at 290 nm as described by Brueske [12]. Tomato leaves (1gm) were homogenized in 3 ml of sodium borate buffer (0.1 M), pH 7.0 containing 0.5 g insoluble polyvinylpolypyrrolidone (PVP) and 0.05 mM phenyl methane sulfonyl fluoride (PMSF). The extract was centrifuged at 8,000 rpm for 15 min at 4^oC. The supernatant was used for enzyme assay. Samples containing 0.2 ml of enzyme extract were incubated with 0.5 ml of borate buffer (0.2 M), pH 8.7, 1.3 ml Distilled water and 1.0 ml of L – phenylalanine (0.1 M) in the same buffer for 30 min at 30^oC. After 30 min the reaction was stopped by adding Trichloroacetic acid (1M). In reference cell, 0.2 ml of enzyme extract was taken along with 0.5 ml of borate buffer, 1.3 ml Distilled water, 1 ml of L – phenylalanine and 0.5 ml of Trichloroacetic acid. The amount of Trans-cinnamic acid synthesized was calculated using a

standard graph. Enzyme activity was expressed on fresh weight basis as µmol $min^{-1}ml^{-1}gm^{-1}$.

Chitinase activity (EC. 3.2.1.14)

The colorimetric assay of chitinase was carried out according to the procedure developed by Monreal and Reese [13]. One gram of tomato leaf was extracted with 3ml of Potassium phosphate buffer (200 mM), pH 6.0 with 2 mM Calcium chloride. The homogenate was centrifuged for 15 min at 8,000 g and the supernatant was used as an enzyme source. The reaction mixture consisted of 2 ml of 1.25 % (w/v) colloidal chitin solution and 0.5 ml of enzyme solution was incubated on a rotary platform at a speed sufficient to keep the chitin in suspension at $25^{O}C$. The reaction was stopped by placing the vial in a boiling water bath for 5 minutes. An aliquot of the supernatant (1.0 ml) was pippetted into a glass reagent tube containing 1.5 ml color reagent and incubated for 5 minutes in boiling water bath. After cooling to room temperature, absorbance at 540 nm was measured using the spectrophotometer. N – acetyl glucosamine (GLcNAc) was used as a standard. The enzyme activity was expressed as µmoles GLcNAc gm^{-1} ml^{-1}.

Catalase activity (EC. 1.11.1.6)

The enzyme activity was evaluated by estimating the residual H_2O_2 in the reaction mixture with the help of $KMnO_4$ titrimetrically [14]. One gram of tomato leaf was extracted with 3 ml of potassium phosphate buffer (0.1 M), pH 7.0. The homogenate was centrifuged for 15 min at 8,000 g and the supernatant was used as an enzyme source. 1 ml of enzyme extract was added to a volumetric flask containing 3 ml of Potassium phosphate buffer (0.1 M), pH 7.0, and 2 ml of H_2O_2 (0.005 M). The mixture was incubated at $20^{o}C$ for 1 min. After 1 min reaction was stopped with the application of 10 ml of H_2SO_4 (0.7 N) and residual H_2O_2 was evaluated by titrating it against $KMnO_4$ (0.01 N). The enzyme activity was evaluated against reference reaction mixture containing enzyme extract in an acidified solution at zero time, using its extinction coefficient of 0.036 $\mu mol^{-1}ml^{-1}$ expressed as $\mu gm\ min^{-1}ml^{-1}$

***In vivo* assay**

The isolate Fusarium oxysporum f. sp. lycopersici, maintained on Potato dextrose broth was used to prepare inoculum suspension following the method of Thakur [15] for in vivo assay. The ten cultivars of tomato selected for the present study were grown in pots. The experimental pots were inoculated with 10 ml of 10 days old inoculum suspension containing mycelia and spores, through soil after the plants reached maturity (60 days), while the control pots were devoid of the fungal inoculum. A standard resistant check (LA 3042) and a susceptible check (Pusa ruby) were included in the in vivo assay to assess the response of the plants to the pathogen. Following incubation, the foliage were scored for external symptoms of disease using a scale of 0 – 4 suggested by Bora [16]. Scale 0= no symptom, 1= symptom on leaves 1-25%, lower leaves yellow, 2= symptom on leaves 26-50%, plants show yellowing or wilting of two leaves more leaves; 3= symptom on leaves 51-75%, plants show vessel browning nearly to the leader shoot, with the most leaves wilt except the leader shoot, 4= symptom on leaves 76-100%, plants show wilt of leaves up to the shoot or died.

Statistical analysis

Each experiment was completely randomized and repeated twice. The statistical package for social sciences was used (version 8.0 for windows, SPSS Inc.). Mann-whitney U- tests and Fisher's linear discriminant analysis were performed to validate the findings by statistical analysis [17].

RESULTS AND DISCUSSION

In vitro assay

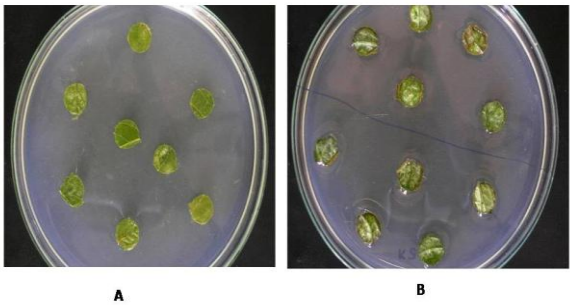

Fig.1 A) Control plate after 24 hrs of incubation showing asymptomic leaf discs B) Test plate after 24 hrs of incubation showing asymptomic and symptomic leaf discs.

Figure 1 shows the effect of fungal culture filtrate on tomato leaves. After 24 hrs of incubation, some cultivars showed chlorosis/fusarosis at the periphery of leaf discs which differed in severity i.e. area of lesion while others were not affected. The disease severity was just discernible at 24 hrs. Therefore, it was further incubated for another 24 hrs.

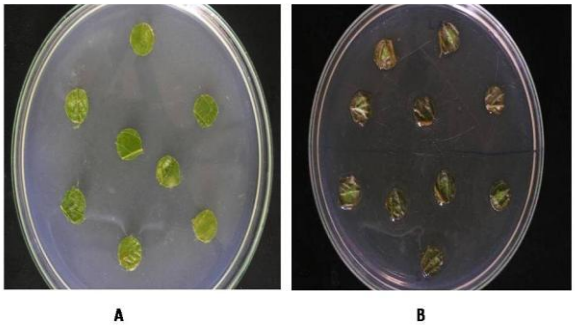

Fig.2 A) Control plate after 48 hrs of incubation showing asymptomic leaf discs B) Test plate after 48 hrs of incubation showing symptomic leaf discs with difference in disease severity.

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After 48 hrs, cultivars which were showing chlorosis after 24 hrs showed significant increase in the symptoms while others which were unaffected revealed either chlorosis at periphery or remained asymptomatic (Figure 2). Incubation for further 24 hrs did not show any change in chlorosis. Test plates in the both figures represents the susceptible cultivars where as resistant cultivar were found to be asymptomic, identical to the control plate.
The cultivars that showed symptoms after 24 hrs of treatment were Pusa ruby, Junagadh ruby, Gujarat local 1, Gujarat local 2, Anand-1 and Maha-2. After 48 hrs, above cultivars showed complete necrosis of the leaf discs while cultivars Heamsona, SS-NC, NDT- 96 and LA3042 remained asymptomatic.

The fungal culture filtrate used was checked for the presence of live spores by microscopic examination and culture method and it was observed that the fungal culture filtrate is devoid of spores. So it is inferred that the leaf discs are not challenged by the spores of the fungus but it is the toxin which actually causes the damage to the leaf discs.

As conventional field studies of resistance to disease are time consuming and destructive, an easy to do procedure was previously developed to differentiate field grown resistant and susceptible cultivars of tomato. Such a procedure involved the *in vitro* treatment of field grown leaves with fungal culture filtrate of *Fusarium oxysporum* f. sp. lycopersici and the measurement of the lesion areas after 48 hrs. A continuous dip method of inoculation was preferred rather than drops or spray inoculation as followed by companioni [17]. Incase of drop or spray inoculation, certain parameters like flat leaf, uniform coverage area, volume of inoculation, wounds on leaf had to be specially taken in to account where as flooding base with Fungal culture filtrate reduces the chances of error. The bioassay was carried out using young and laterally positioned leaf from the first node of the plant, because leaf position and age are critical factors that decide the reproducibility of a bioassay [6]. The continuous dip method proved to be a reliable bioassay for determining susceptible and resistant cultivars of tomato, since very rapid disease symptom was observed which could not have been achieved with the standard leaf inoculation methods.

Rate of disease development was much greater on detached leaves than whole plants with high lesion coverage occurring with a short time period compared to whole plant [18]. The interpretation of field data is difficult because the expression of the disease is almost certainly affected by climatic and other environmental conditions and several years are needed to confidently evaluate the resistance [19]. A detached leaf assay was intentionally practiced to minimize the space and error due to artificial wounding. The evaluation phase of bioassay took only two days in comparison to drop/spray inoculation which takes 3-5 days [6].

Inoculum's aggressiveness

Moreover, the expression of plant resistance mechanisms depends on the conditions and timings of plant inoculation and aggressiveness of pathogen [19]. Inoculum's aggressiveness was also awarded with an equal importance to minimize the evaluation phase and sterility condition. Initially leaf was inoculated with 1 X FCF which resulted in lesion after five days of incubation in comparison to 15 X in two days. In terms of simplicity and reproducibility base spreading method were preferred over drop/spray method.

Quantification of disease severity

There was a significant difference in the chlorophyll reduction indices among the cultivars (Table.1). Cultivars Heamsona, LA-3042, NDT-96 and SS-NC had significantly less chlorophyll reduction at P=0.003 (*U*-Test) than any other cultivars. Anand-1, Pusa Ruby, Junagadh Ruby, Gujarat local 1, Gujarat local 2 and Maha-2 showed significantly higher chlorophyll reduction at P=0.003(*U*-Test). The results obtained by chlorophyll estimation were reproducible.

Nelson [6] proposed a method for assessing the disease severity of tomato to late blight using the scanned digital images of the affected leaves. However it was a time consuming operation. Although scanning the leaves did not take much time, incorporating the images in to the analysis software and doing preliminary calibration required many hours to make measurement for only a few leaves. Moreover the severity of fusarosis does not always manifest itself in the form of necrosis or wilting, especially at early stage of plant growth, but can affect the process of photosynthesis [20]. Chlorophyll fluorescence is an indicator of photosynthetic performance of plants [21]. Measurement of chlorophyll fluorescence indicated a negative effect of pathogens on the photosynthetic process [22]. So for the fastest method of accessing disease severity, chlorophyll level study was worth to be evaluated. When cultivars were classified according to chlorophyll level data suggest that as the plant susceptible chlorophyll level decreases. Moreover the variation obtained was significant statistically at P=0.003(U-Test) compared to companioni [17] with insignificant result for chlorophyll level.

Biochemical marker evaluation

To further confirm the sensitivity of bioassay, baseline evaluation of specific enzymes were carried out. Table-1 represents the concentration of each enzyme at zero hrs in the resistant and susceptible cultivars. It was marked that, the cultivars showing less chlorophyll reduction showed higher enzyme activity.

The susceptible cultivars Pusa Ruby, Junagadh Ruby, Gujarat local 1, Maha2, Gujarat local 2 and Anand 1 showed remarkably less enzyme activity at P=0.010(*U*-Test) of the enzymes assayed and a lower total phenolics content compared to resistant cultivars Heamsona, LA-3042, SS-NC and NDT96 with higher activity at P=0.010(*U*-Test) of all enzymes.

In this work, consideration was also given to some of the general biochemical substances regarded to be involved in resistance, such as phenolics, PAL, chitinase, β-1, 3 glucanase, peroxidase, poly phenol oxidase and catalase. PAL is the first enzyme of phenylpropanoid metabolism in higher plants and it plays a significant role in regulating the accumulation of phenolics, phytoalexins and lignins, the three key factors responsible for disease resistance [23]. A direct role of chitinase and β-1,3 Glucanase in defence of plants against pathogen have been proposed because substrate for these enzymes are major component of the cell walls of many fungi [24, 25]. PPO is a copper containing enzyme which oxidizes phenolics to highly toxic quinines and involved in the terminal oxidation of diseased plant tissue which was attributed for its role in disease resistance [26].

Table 1. *In vitro* assessment of disease severity and biochemical parameters

Cultivars	Phenotypic markers							
	Total chlorophyll reduction (%)	Phenyl ammonia lyase(Units/min/g/ml)	Peroxidase (Units/min/g/ml)	Chitinase (Units/ml/g)	B-glucanase (Units/ml/min)	Poly phenol oxidase (Units/ml/g)	Catalase (µg/min/ml)	Total phenol (µg/g)
Pusa ruby	38	0.3	1.9	1.36	0.11	0.08	8.49	25.0
Junagadh ruby	42	0.6	1.9	1.47	0.03	0.05	16.9	32.4
Local 1	46	0.4	1.8	1.50	0.05	0.04	16.9	33.5
Local 2	45	0.5	1.9	1.49	0.06	0.06	8.49	35.4
Maha-2	42	0.5	0.7	1.52	0.03	0.09	8.49	33.9
Anand-1	35	0.5	1.4	1.56	0.10	0.04	16.9	36.0
Heamsona	5.0	4.7	7.8	1.92	0.63	0.38	84.9	73.5
NDT-96	4.0	5.4	8.4	2.53	0.81	0.44	93.3	96.7
SS-NC	2.0	5.5	8.6	1.98	0.62	0.41	85.0	82.5
LA 3042	0.0	4.4	6.7	1.96	0.60	0.32	101	76.7
(FLD) Function for susceptible cultivars	60.082	-189.362	43.090	1236.847	-200.347	-455.732	-1.650	-20.719
(FLD) Function for resistant cultivars	-9.746	569.325	-203.435	-550.746	-2741.995	-516.981	9.504	26.560

In vivo assay

The *in vivo* assay on the tomato cultivars carried out in presence and absence of pathogen (Table. 2) showed positive correlation with the *in vitro* enzyme analysis data obtained through detached leaf assay. And-1, Junagadh ruby, Gujarat local 1, Gujarat local 2 and Maha-2 cultivars showed symptoms after 6 days of inoculation and wilted completely in four weeks as in the susceptible check 'Pusa ruby'. Similar correlation was obtained incase of highly resistant cultivars in which the cultivars NDT-96, SS-NC and Heamsona remained asymptotic with resistant check 'LA 3042'.

Table 2. Rating of infection caused by *Fusarium oxysporum* f. sp. *lycopersici* on different cultivars of tomato at different time intervals (*In vivo* study)

Cultivars	Days after inoculation										
	1	2	3	6	9	12	15	18	21	24	27
Pusa ruby	0	0	0	1	1	2	2	3	3	4	4
Junagadh Ruby	0	0	0	1	1	2	2	3	3	4	4
Local 1	0	0	0	1	1	2	2	3	3	4	4
Local 2	0	0	0	1	1	2	2	3	3	4	4
Maha-2	0	0	0	1	1	2	2	3	3	4	4
Anand 1	0	0	0	1	1	2	2	3	3	4	4
Heamsona	0	0	0	0	0	0	0	0	0	0	0
SS-NC	0	0	0	0	0	0	0	0	0	0	0
NDT 96	0	0	0	0	0	0	0	0	0	0	0
LA 3042	0	0	0	0	0	0	0	0	0	0	0

Statistical evaluation

The data obtained from chlorophyll estimation and biochemical analysis were scrutinized through Mann-Whitney *U*-Tests and Fisher's linear discrimination analysis. Table 1 represents the statistical analysis of two classes for discriminant analysis by fisher's model. The table shows that the data were significant to discriminate them in to two suitable classes and the cultivars were correctly classified.

The data obtained were subjected to a statistical package consisting of *T*-test, Mann-Whitney *U*-Test and Fisher's linear discriminant model [17] for the discrimination of susceptible and resistant cultivars. The results obtained were most significant.

The proposed method requires less space, because of the use of leaf cuttings (discs) rather than whole leaf. In a single run many representatives of particular cultivars can be scored compared to whole leaf method. The sensitivity and rapidness could not have been achieved with such ease with standard inoculation and image analysis methods. Therefore applying this sensitive screening technique to encompass screening procedure for wilt causing fungi would be beneficial.

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